

1 **Gene expression heterogeneity during brain development and aging: temporal  
2 changes and functional consequences**

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4 Ulaş İşıldak<sup>1</sup>, Mehmet Somel<sup>1</sup>, Janet M. Thornton<sup>2</sup>, Handan Melike Dönertaş<sup>2\*</sup>  
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6 <sup>1</sup>Department of Biological Sciences, Middle East Technical University, 06800, Ankara, Turkey.  
7 <sup>2</sup>European Molecular Biology Laboratory, European Bioinformatics Institute, Wellcome Trust Genome  
8 Campus, Hinxton, Cambridge, CB10 1SD, UK.  
9 \*correspondence: [donertas.melike@gmail.com](mailto:donertas.melike@gmail.com)

10  
11 **ORCID**  
12 *Ulaş İşıldak* - <https://orcid.org/0000-0001-6497-6254>  
13 *Mehmet Somel* - <https://orcid.org/0000-0002-3138-1307>  
14 *Janet M. Thornton* - <http://orcid.org/0000-0003-0824-4096>  
15 *Handan Melike Dönertaş* - <http://orcid.org/0000-0002-9788-6535>  
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18 **Abstract**

19 Cells in largely non-mitotic tissues such as the brain are prone to stochastic (epi-)genetic alterations  
20 that may cause increased variability between cells and individuals over time. Although increased inter-  
21 individual heterogeneity in gene expression was previously reported, whether this process starts during  
22 development or if it is restricted to the aging period has not yet been studied. The regulatory dynamics  
23 and functional significance of putative aging-related heterogeneity are also unknown. Here we address  
24 these by a meta-analysis of 19 transcriptome datasets from diverse human brain regions. We observed  
25 a significant increase in inter-individual heterogeneity during aging (20+ years) compared to postnatal  
26 development (0 to 20 years). Increased heterogeneity during aging was consistent among different  
27 brain regions at the gene level and associated with lifespan regulation and neuronal functions. Overall,  
28 our results show that increased expression heterogeneity is a characteristic of aging human brain, and  
29 may influence aging-related changes in brain functions.

30  
31 **Keywords:** aging, development, gene expression, transcriptome, heterogeneity, human, brain

32 Aging is a complex process characterized by a gradual decline in maintenance and repair mechanisms,  
33 accompanied by an increase in genetic and epigenetic mutations, and oxidative damage to nucleic  
34 acids, protein and lipids<sup>1,2</sup>. The human brain experiences dramatic structural and functional changes in  
35 the course of aging. These include decline in gray matter and white matter volumes<sup>3</sup>, increase in axonal  
36 bouton dynamics<sup>4</sup> and reduced synaptic plasticity, all processes that may be associated with decline in  
37 cognitive functions<sup>5</sup>. Changes during brain aging are suggested to be a result of stochastic processes,  
38 unlike changes associated with postnatal neuronal development that are known to be primarily  
39 controlled by adaptive regulatory processes<sup>6-8</sup>. The molecular mechanisms underlying age-related  
40 alteration of regulatory processes and eventually leading to aging-related phenotypes, however, are  
41 little understood.

42

43 Over the past decade, a number of transcriptome studies focusing on age-related changes in human  
44 brain gene expression profiles were published<sup>2,9-12</sup>. These studies report aging-related differential  
45 expression patterns in many functions, including synaptic functions, energy metabolism, inflammation,  
46 stress response, and DNA repair. By analyzing age-related change in gene expression profiles in  
47 diverse brain regions, we previously showed that for many genes, gene expression changes occur in  
48 opposite directions during postnatal development (pre-20 years of age) and aging (post-20 years of  
49 age), which may be associated with aging-related phenotypes in healthy brain aging<sup>13</sup>. While different  
50 brain regions are associated with specific, and often independent, gene expression profiles<sup>9,10,12</sup>, these  
51 studies also show that age-related alteration of gene expression profiles during aging is a widespread  
52 effect across different brain regions.

53

54 One of the suggested effects of aging is increased variability between individuals and somatic cells,  
55 which has been previously reported by several studies. Some of these studies find an increase in age-  
56 related heterogeneity in heart, lung and white blood cells of mice<sup>14-16</sup>, *Caenorhabditis elegans*<sup>17</sup>, and  
57 human twins<sup>18</sup>. A study analysing microarray datasets from different tissues of humans and rats also  
58 reported an increase in age-related heterogeneity in expression as a general trend<sup>19</sup>, although this study  
59 found no significant consistency across datasets, nor any significant enrichment in functional gene  
60 groups. That said, the generality of increase in expression heterogeneity remains unresolved. For  
61 instance, Viñuela et al. find more decrease than an increase in heterogeneity in human twins<sup>20</sup> and  
62 Ximerakis et al. show the direction of the heterogeneity change depends on cell type in aging mice  
63 brain<sup>21</sup>. Using GTEx data covering different brain regions (20 to 70 years of age), Brinkmeyer-Langford  
64 et al. identify a set of differentially variable genes between age groups, but they do not observe  
65 increased heterogeneity at old age<sup>22</sup>. Meanwhile, another study performing single-cell RNA sequencing  
66 of human pancreatic cells, identifies an increase in transcriptional heterogeneity and somatic mutations  
67 with age<sup>23</sup>. A meta-analysis also suggested more shared expression patterns during development than  
68 in aging, implying an increase in inter-individual variability<sup>13</sup>. Likewise, a prefrontal cortex transcriptome

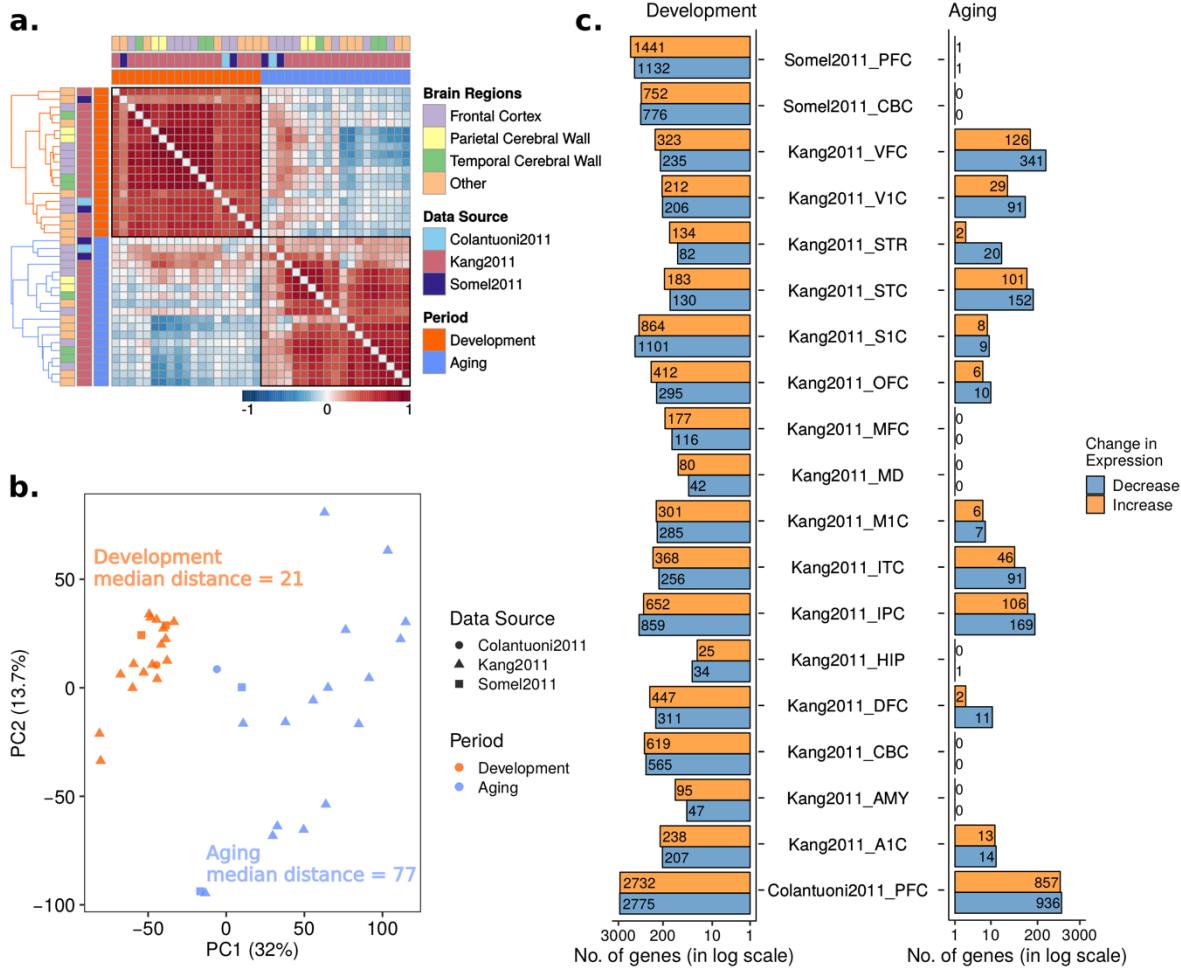
69 analysis we recently conducted revealed a weak increase in age-dependent heterogeneity at the gene,  
70 transcriptome and pathway levels, irrespective of the preprocessing methods<sup>24</sup>.

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72 Whether age-related increase in heterogeneity is a universal phenomenon thus remains contentious.  
73 Furthermore, where it can be detected, whether this is a time-dependent process that starts at the  
74 beginning of life or whether this increase and its functional consequences are only seen after  
75 developmental processes are completed, have not yet been explored. In this study, we retrieved  
76 transcriptome data from independent studies covering the whole lifespan, including data from diverse  
77 brain regions, and conducted a comprehensive analysis to identify the prevalence of age-related  
78 heterogeneity changes in human brain aging compared with those observed during postnatal  
79 development. We confirmed that increased age-related heterogeneity is a consistent trend in the human  
80 brain transcriptome during aging but not during development, and it is associated with the pathways  
81 and biological functions that are related to longevity and neuronal function.

82  
83 **Results**  
84 To investigate how heterogeneity in gene expression changes with age, we used 19 published  
85 microarray datasets from three independent studies. Datasets included 1,010 samples from 17 different  
86 brain regions of 298 individuals whose ages ranged from 0 to 98 years (Supplementary Table S1, Fig.  
87 S1). In order to analyze the age-related change in gene expression heterogeneity during aging  
88 compared to the change in development, we divided datasets into two subsets as development (0 to  
89 20 years of age,  $n = 441$ ) and aging (20 to 98 years of age,  $n = 569$ ). We used the age of 20 to separate  
90 pre-adulthood and adulthood based on commonly used age intervals in earlier studies (see Methods).  
91 For the analysis, we focused only on the genes for which we have a measurement across all datasets  
92 ( $n = 11,137$ ).

93  
94 **Age-related change in gene expression levels**  
95 To quantify age-related changes in gene expression, we used a linear model between gene expression  
96 levels and age (see Methods, Supplementary Fig. S2). We transformed the ages to the fourth root scale  
97 before fitting the model as it provides relatively uniform distribution of sample ages across the lifespan,  
98 but we also confirmed that different age scales yield quantitatively similar results (see Supplementary  
99 Fig. S3). We quantified expression change of each gene in aging and development periods separately  
100 and considered regression coefficients from the linear model ( $\beta$  values) as a measure of age-related  
101 expression change (Supplementary Fig. S4).

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**Figure 1.** Age-related change in gene expression during postnatal development and aging. (a) Spearman correlations among age-related expression changes ( $\beta$  values) across datasets. The color of the squares indicates if the correlation between the corresponding pair of datasets (across  $\beta$  values of 11,137 common genes) is positive (red) or negative (blue), while darker color specifies a stronger correlation. Diagonal values were removed in order to enhance visuality. Annotation rows and columns indicate data source, brain region and period of each dataset. Hierarchical clustering was performed for each period separately (color of the dendrogram indicates periods) to determine the order of datasets. (b) Principal component analysis (PCA) of age-related expression changes during aging and development. The analysis was performed on age-related expression change values of 11,137 common genes among all 38 datasets. The values of the first principal component on the x-axis and second principal component on the y-axis were drawn, where the values in the parenthesis indicate the variation explained by the corresponding principal component. Median Euclidean pairwise distances among development and aging datasets calculated using PC1 and PC2 were annotated on the figure. Different shapes show different data sources and colors show development (dark orange) and aging (blue) (c) Number of significant (FDR corrected  $p < 0.05$ ) gene expression changes in development (left panel) and aging (right panel). The x-axis shows the number of genes in the log scale. The color of the bars shows the direction of change, decrease (steel gray), and increase (orange). The exact number of genes are also displayed on the plot.

121 We first analyzed similarity in age-related expression changes across datasets by calculating pairwise  
122 Spearman's correlation coefficients among the  $\beta$  values (Figure 1a). Both development (median  
123 correlation coefficient = 0.56, permutation test  $p < 0.001$ , Supplementary Fig. S5) and aging datasets  
124 (median correlation coefficient = 0.43, permutation test  $p = 0.003$ , Supplementary Fig. S5) showed  
125 moderate correlation with the datasets within the same period. Although the difference between dataset  
126 correlations within development and aging datasets was not significant (permutation test  $p = 0.1$ ,  
127 Supplementary Fig. S6), weaker consistency during aging may reflect the stochastic nature of aging,  
128 causing increased heterogeneity between aging datasets.

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130 The principal component analysis (PCA) of age-related expression changes ( $\beta$ ) revealed distinct  
131 clusters of development and aging datasets (Figure 1b). Moreover, aging datasets were more dispersed  
132 than development datasets (median pairwise Euclidean distances between PC1 and PC2 were 77 for  
133 aging and 21 for development), which may again reflect stochasticity in gene expression change during  
134 aging and can indicate more heterogeneity among different brain regions or datasets during aging than  
135 in development.

136

137 We next identified genes showing significant age-related expression change (FDR-corrected  $p < 0.05$ ),  
138 for development and aging datasets separately (Figure 1c). Development datasets showed more  
139 significant changes compared to aging (permutation test  $p = 0.003$ , Supplementary Fig. S6), which may  
140 again indicate higher expression variability among individuals during aging. The direction of change in  
141 development was mostly positive (14 datasets with more positive and 5 with more negative), whereas  
142 in aging datasets, we observed more genes with a decrease in expression level (13 datasets with more  
143 genes decreasing expression and 5 with no significant change, and 1 with an equal number of positive  
144 and negative changes).

145

#### 146 **Age-related change in gene expression heterogeneity**

147 To assess age-related change in heterogeneity, we obtained the unexplained variance (residuals) from  
148 the linear models used to calculate the change in gene expression level. For each gene in each dataset,  
149 we separately calculated Spearman's correlation coefficient ( $\rho$ ) between the absolute value of residuals  
150 and age, irrespective of whether the gene shows a significant change in expression (see Methods,  
151 Supplementary Fig. S2). We considered  $\rho$  values as a measure of heterogeneity change, where positive  
152 values mean an increase in heterogeneity with age. We also repeated this approach using loess  
153 regression instead of a linear model between expression level and age, and found high correspondence  
154 between  $\rho$  values based on linear and loess regression models (Supplementary Fig. S7). Still, loess  
155 regression was more sensitive to the changes in sample sizes and parameters and we therefore  
156 continued downstream analyses with the  $\rho$  estimates based on the residuals from the linear model.

157

158 We next asked if datasets show similar  $\rho$ , *i.e.* age-related changes in heterogeneity, by calculating  
159 pairwise Spearman's correlation between pairs of datasets, across shared genes (Figure 2a). Unlike  
160 the correlations among expression level changes,  $\rho$  values did not show a higher consistency during  
161 development. In fact, although the difference is not significant (permutation test  $p = 0.2$ , Supplementary  
162 Fig. S6), the median value of the correlation coefficients was higher in aging (median correlation  
163 coefficient = 0.21, permutation test  $p = 0.24$ , Supplementary Fig. S5), than in development (median  
164 correlation coefficient = 0.11, permutation test  $p = 0.25$ , Supplementary Fig. S5).

165

166 A principal component analysis (PCA) showed that, like expression change, heterogeneity change with  
167 age can also differentiate aging datasets from development (Figure 2b). Similar to the pairwise  
168 correlations (Figure 2a), aging datasets clustered more closely than development datasets (median  
169 pairwise Euclidean distances between PC1 and PC2 are 41 and 44 for aging and development,  
170 respectively). Both observations imply more similar changes in heterogeneity during aging.

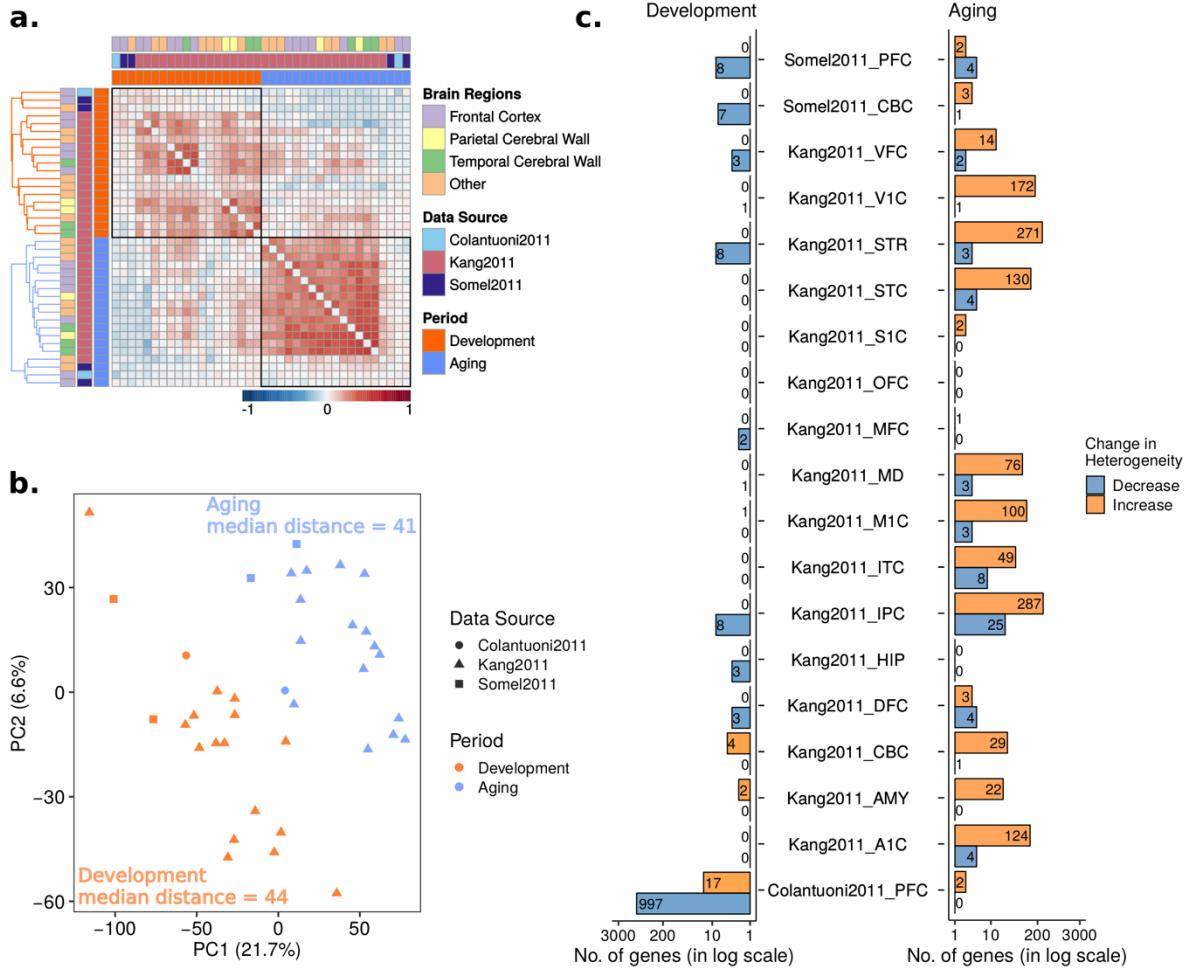
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172 Using the  $p$ -values from Spearman's correlation between age and the absolute value of residuals for  
173 each gene, we then investigated the genes showing a significant change in heterogeneity during aging  
174 and development (FDR corrected  $p$ -value  $< 0.05$ ). We found almost no significant change in  
175 heterogeneity during development, except for the Colantuoni2011 dataset, for which we have high  
176 statistical power due to its large sample size. In aging datasets, on the other hand, we observed more  
177 genes with significant changes in heterogeneity (permutation test  $p = 0.06$ , Supplementary Fig. S6) and  
178 the majority of the genes with significant changes in heterogeneity tended to increase in heterogeneity  
179 (Figure 2c). However, the genes showing a significant change did not overlap across aging datasets  
180 (Supplementary Fig. S8).

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182 Nevertheless, our analyses indicated relatively more consistent heterogeneity changes among datasets  
183 in aging compared to development, implying that heterogeneity change could be a characteristic linked  
184 to aging (see Discussion).

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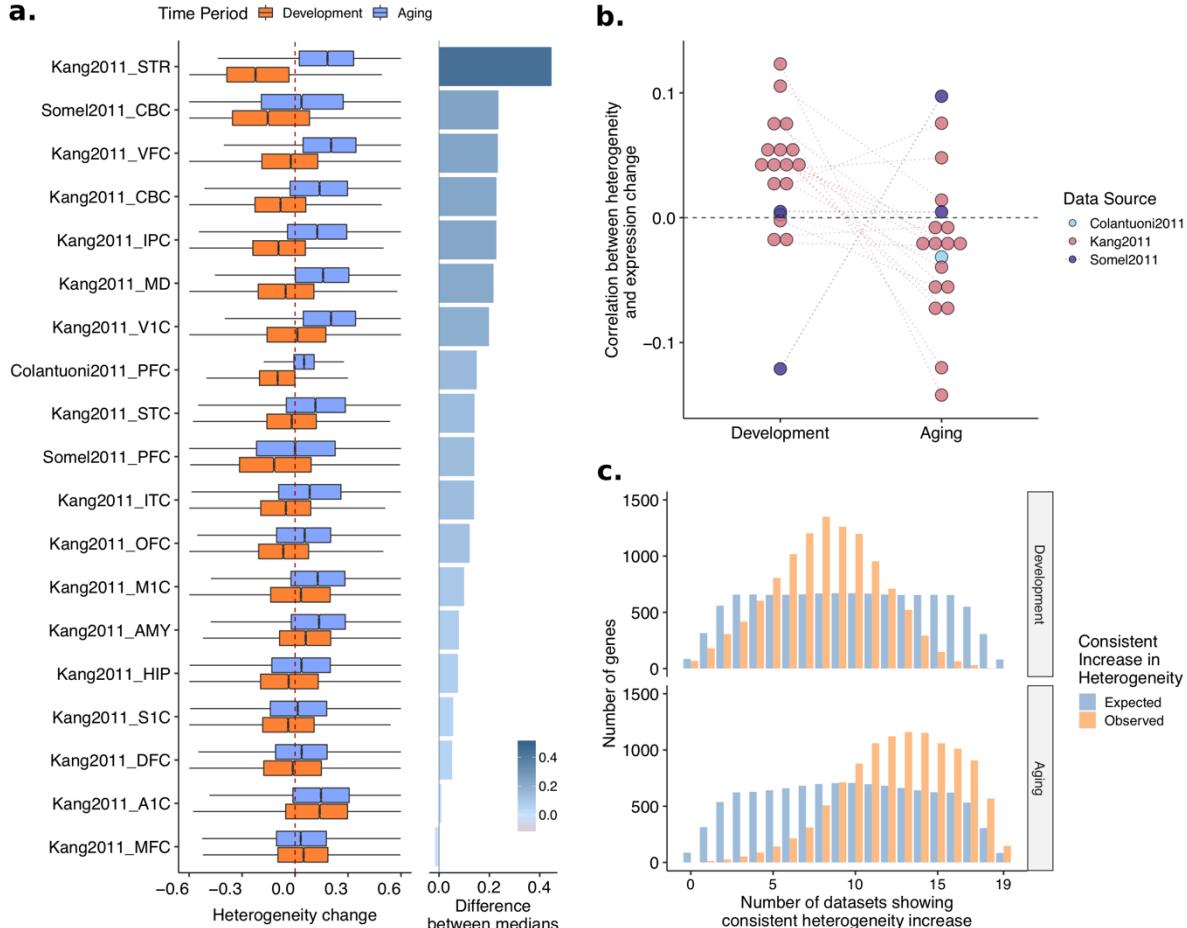
187 **Figure 2. Age-related change in gene expression heterogeneity during development and aging.** The procedures  
 188 are similar to those in Figure 1, except, age-related heterogeneity changes ( $\rho$  values) were used instead of  
 189 expression changes ( $\beta$  values). (a) Spearman correlations among age-related heterogeneity changes ( $\rho$  values)  
 190 across datasets. (b) Principal component analysis (PCA) of heterogeneity change with age. (c) The number of  
 191 genes showing significant heterogeneity change in aging and development.

192

### 193 Consistent increase in heterogeneity during aging

194 As our previous analyses suggested age-related changes in heterogeneity can differentiate  
 195 development from aging and show more similarity during aging, we sought to characterize the genes  
 196 displaying such changes. Since the significance of the changes is highly dependent on the sample size,  
 197 instead of focusing on significant genes identified within individual datasets, we leveraged upon the  
 198 availability of multiple datasets and focused on their shared trends, capturing weak but reproducible  
 199 trends across multiple datasets. Consequently, we used the level of consistency in age-related  
 200 heterogeneity change across datasets to sort genes.

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203 **Figure 3.** (a) Boxplots, showing distributions of age-related heterogeneity changes ( $\rho$  values) of 11,1137 common  
 204 genes for each dataset and period separately. The dotted red line (vertical line at  $x = 0$ ) reflects no change in  
 205 heterogeneity. The difference between median heterogeneity change in aging and development is given as a bar  
 206 plot on the right panel. Datasets are ordered by the differences in median heterogeneity changes in aging and  
 207 development. (b) The relationship between expression and heterogeneity change with age. Spearman correlation  
 208 analysis was performed between age-related expression changes ( $\beta$  values) and age-related heterogeneity  
 209 changes ( $\rho$  values) of 11,137 common genes, separately for each dataset. The dotted gray line at  $y = 0$  reflects no  
 210 correlation between expression and heterogeneity. (c) Expected and observed consistency in the heterogeneity  
 211 change across datasets in development and aging. There is a significant shift toward heterogeneity increase in  
 212 aging (permutation test  $p < 10^{-7}$ ) (lower panel), while there is no significant consistency in either direction in  
 213 development (upper panel). The expected distribution is constructed using a permutation scheme that accounts  
 214 for the dependence among datasets and is more stringent than random permutations (see Supplementary Fig. S10  
 215 for details).

216

217 We first examined profiles of age-related heterogeneity change in aging and development. Among  
 218 aging datasets 18/19 showed more increase than decrease in heterogeneity with age (median  $\rho > 0$ ,  
 219 i.e. higher numbers of genes with increase), while the median heterogeneity change in one dataset was  
 220 zero. In development, on the other hand, only 5/19 datasets showed more increase in heterogeneity,

221 while the remaining 14/19 datasets showed more decrease with age (median  $\rho < 0$ ) (Figure 3a). The  
222 age-related change in heterogeneity during aging was significantly higher than during development  
223 (permutation test  $p < 0.001$ , Supplementary Fig. S6). We also checked if there is a relationship between  
224 changes in heterogeneity during development and during aging (e.g. if those genes that decrease in  
225 heterogeneity tend to increase in heterogeneity during aging) but did not find any significant trend  
226 (Supplementary Fig. S9).

227

228 A potential explanation why we see different patterns of heterogeneity change with age in development  
229 and aging could be the accompanying changes in the expression levels, as it is challenging to remove  
230 dependence between the mean and variance. To address this possibility, we first calculated  
231 Spearman's correlation coefficient between the changes in heterogeneity ( $\rho$  values) and expression  
232 ( $\beta$  values), for each dataset. Overall, all datasets had values close to zero, suggesting the association  
233 is not strong. Surprisingly, we saw an opposing profile for development and aging; while the change in  
234 heterogeneity and expression were positively correlated in development, they showed a negative  
235 correlation in aging (Figure 3b).

236

237 Having observed both a tendency to increase and a higher consistency in heterogeneity change during  
238 aging, we asked which genes show consistent increase in heterogeneity across datasets in aging and  
239 development. We therefore calculated the number of datasets with an increase in heterogeneity during  
240 development and aging for each gene (Figure 3c). To calculate significance and expected consistency,  
241 while controlling for dataset dependence, we performed 1,000 random permutations of individuals' ages  
242 and re-calculated the heterogeneity changes (see Methods). In development, there was no significant  
243 consistency in heterogeneity change in either increase or decrease. During aging, however, there was  
244 a significant signal of consistent heterogeneity increase, *i.e.* more genes showed consistent  
245 heterogeneity increase across aging datasets than randomly expected (Figure 3c, lower panel). We  
246 identified 147 common genes with a significant increase in heterogeneity across all aging datasets  
247 (permutation test  $p < 0.001$ , Supplementary Table S2). Based on our permutations, we estimated that  
248 84/147 genes could be expected to have consistent increase just by chance, suggesting only ~40%  
249 true positives. In development, in contrast, there was no significant consistency in heterogeneity change  
250 in either direction (increase or decrease). Nevertheless, comparing the consistency in aging and  
251 development, there was an apparent shift towards a consistent increase in aging – even if we cannot  
252 confidently report the genes that become significantly more heterogeneous with age across multiple  
253 datasets.

254

## 255 **Heterogeneity Trajectories**

256 We next asked if there are specific patterns of heterogeneity change, *e.g.* increase only after a certain  
257 age. We used the genes with a consistent increase in heterogeneity with age during aging ( $n = 147$ ) to  
258 explore the trajectories of heterogeneity change (Figure 4). Genes grouped with k-means clustering

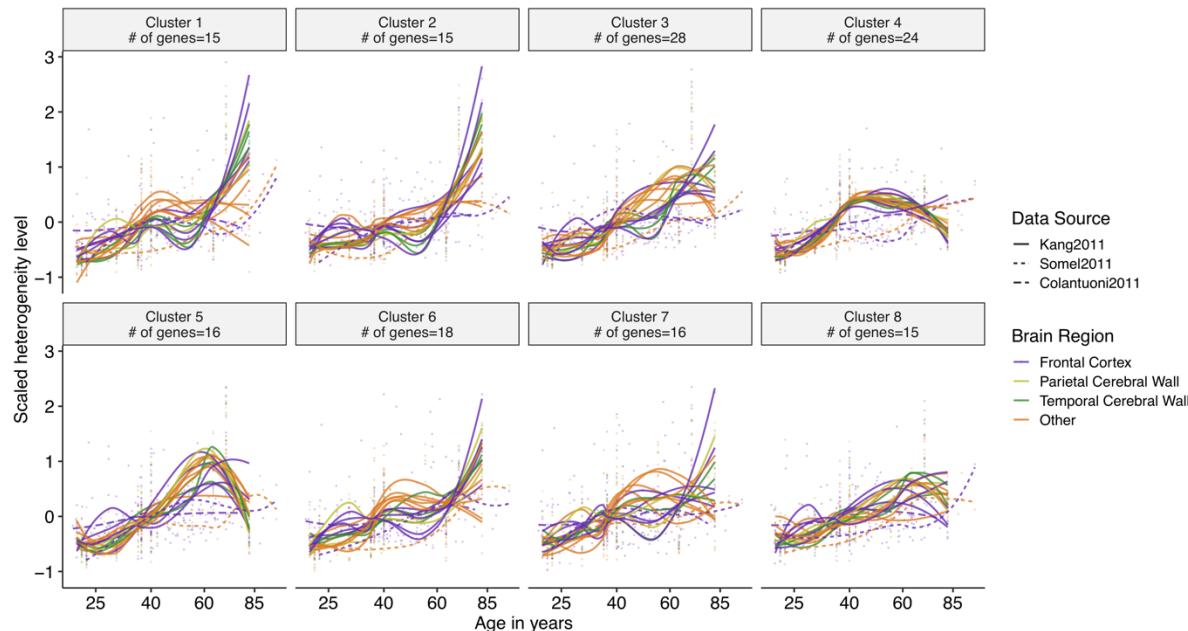
259 revealed three main patterns of heterogeneity increase (Supplementary Table S2): i) genes in clusters  
260 3 and 7 show noisy but a *steady increase* throughout aging, ii) genes in clusters 4, 5 and 8 show  
261 *increase in early aging but a later slight decrease*, revealing a reversal (up-down) pattern, and iii) the  
262 remaining genes in cluster 1, 2 and 6 *increase in heterogeneity dramatically after the age of 60*. We  
263 next asked if these genes have any consistent heterogeneity change pattern in development  
264 (Supplementary Fig. S11), but most of the clusters showed no or only weak age-related changes during  
265 development. We also analyzed the accompanying changes in mean expression levels for these  
266 clusters. Except for cluster 1, which shows a decrease in expression level at around the age of 60 and  
267 then shows a dramatic increase, all clusters show a steady scaled mean expression level at around  
268 zero, *i.e.* different genes in a cluster show different patterns (Supplementary Fig. S12).

269

270 We further tested the genes showing dramatic heterogeneity increase after the age of 60 (clusters 1, 2  
271 and 6) for association with Alzheimer's Disease, as the disease incidence increases after 60<sup>25</sup> as well;  
272 however, we found no evidence for such an association (see Supplementary Fig. S13).

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276 **Figure 4.** Clusters of genes showing a consistent heterogeneity increase in aging ( $n = 147$ ). Clustering was  
277 performed based on patterns of the change in heterogeneity, using the k-means clustering method (see Methods).  
278 The x- and y-axes show age and heterogeneity levels, respectively. Mean heterogeneity change for the genes in  
279 each cluster was drawn by spline curves. The colors and line-types of curves specify different brain regions and  
280 data sources, respectively.

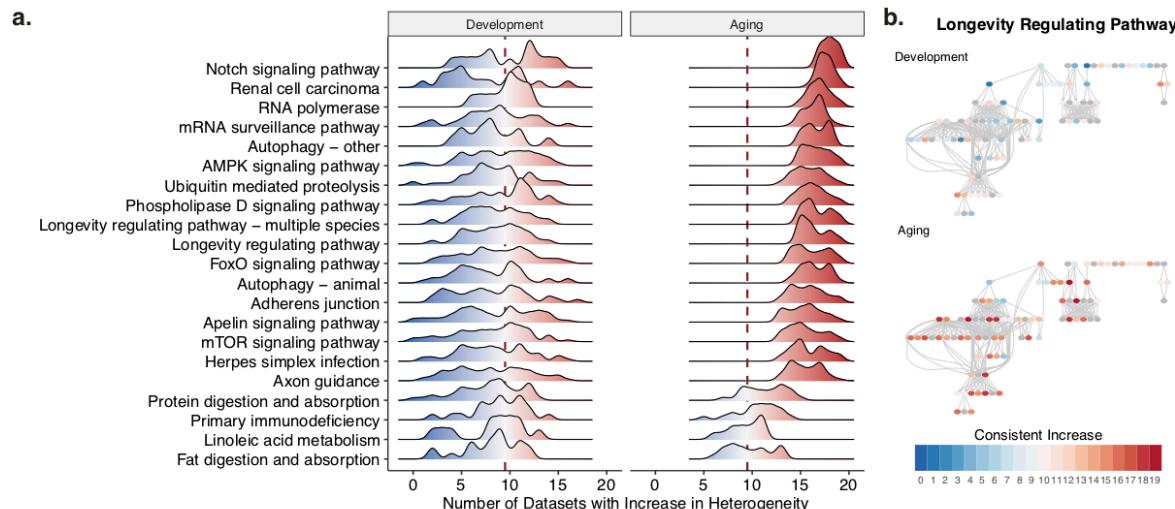
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## 282 Functional analysis

283 To examine the functional associations of heterogeneity changes with age, we performed gene set  
284 enrichment analysis using KEGG pathways<sup>26</sup>, Gene Ontology (GO) categories<sup>27,28</sup>, Disease Ontology

285 (DO) categories<sup>29</sup>, Reactome pathways<sup>30</sup>, transcription factor (TF) targets (TRANSFAC)<sup>31</sup>, and miRNA  
286 targets (MiRTarBase)<sup>32</sup>. Specifically, we rank-ordered genes based on the number of datasets that  
287 show a consistent increase in heterogeneity and asked if the extremes of this distribution are associated  
288 with the gene sets that we analyzed. There was no significant enrichment for any of the functional  
289 categories and pathways for the consistent changes in development. The significantly enriched KEGG  
290 pathways for the genes that become consistently heterogeneous during aging included multiple KEGG  
291 pathways known to be relevant for aging, including the longevity regulating pathway, autophagy<sup>33</sup>,  
292 mTOR signaling<sup>34</sup> and FoxO signaling<sup>35</sup> (Figure 5a). Among the pathways with a significant association  
293 (listed in Figure 5a), only protein digestion and absorption, primary immunodeficiency, linoleic acid  
294 metabolism, and fat digestion and absorption pathways had negative enrichment scores, meaning  
295 these pathways were significantly associated with the genes having the least number of datasets  
296 showing an increase. However, it is important to note that this does not mean these pathways have a  
297 decrease in heterogeneity as the distribution of consistent heterogeneity levels is skewed (Figure 3c,  
298 lower panel). We also calculated if the KEGG pathways that we identified are particularly enriched in  
299 any of the heterogeneity trajectories we identified. Although we lack the necessary power to test the  
300 associations statistically due to small number of genes, we saw that i) group 1, which showed a stable  
301 increase in heterogeneity, is associated more with the metabolic pathways and mRNA surveillance  
302 pathway, ii) group 2, which showed first an increase and a slight decrease at later ages, is associated  
303 with axon guidance, mTOR signaling, and phospholipase D signaling pathways, and iii) group 3, which  
304 showed a dramatic increase after age of 60, is associated with autophagy, longevity regulating pathway  
305 and FoxO signaling pathways. The full list is available as Supplementary Figure S14.

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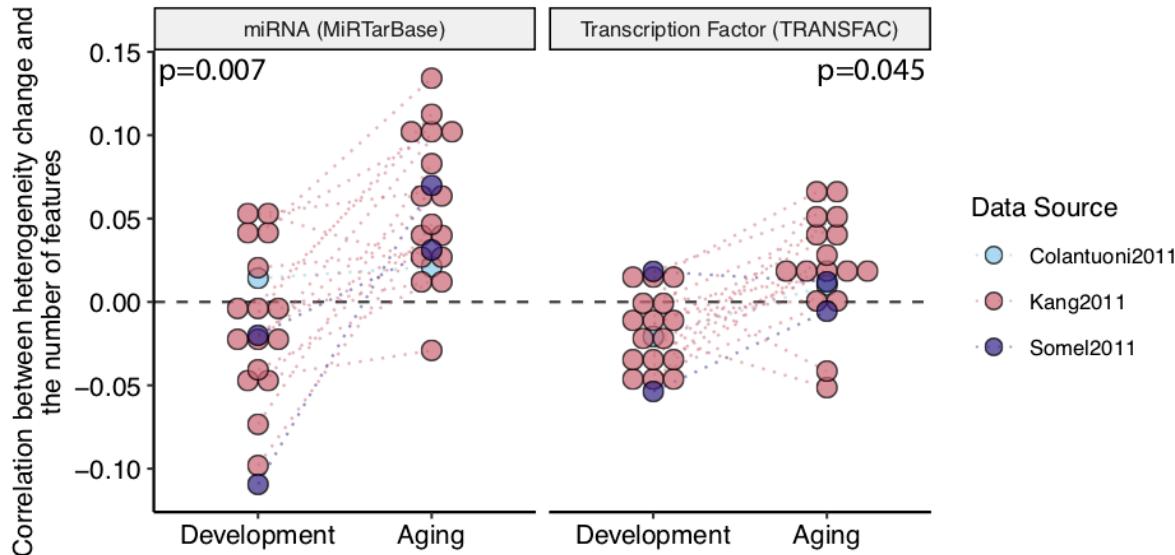
308 **Figure 5.** Functional analysis of consistent heterogeneity changes. (a) Distribution of consistent heterogeneity  
309 increase for the significantly enriched KEGG pathways, in development and aging. x- and y-axes show the number  
310 of datasets with a consistent increase and the density for each significant pathway, respectively. The dashed red  
311 line shows  $x = 9.5$ , which is the middle point for 19 datasets, representing no tendency to increase or decrease.  
312 Values higher than 9.5, shown with red color, indicate an increase in heterogeneity while values lower than 9.5,

313 shown with blue color, indicate a decrease in heterogeneity and the darkness shows the consistency in change  
314 across datasets. b) The longevity regulating pathway (KEGG Pathway ID: hsa04211), exemplifying the distribution  
315 of the genes (circles), their heterogeneity across datasets (color – the same color scheme as panel (a)), and their  
316 relationship in the pathway (edges). More detailed schemes for all significant pathways with the gene names are  
317 given as SI.

318  
319 The distribution of consistent heterogeneity in development and aging also showed a clear difference.  
320 The pathway scheme for the longevity regulating pathway (Figure 5b), colored based on the number of  
321 datasets with a consistent increase, shows how particular genes compare between development and  
322 aging. The visualizations for all significant pathways, including the gene names are given in the  
323 Supplementary Information. Other significantly enriched gene sets, including GO, Reactome, TF and  
324 miRNA sets, are included as Supplementary Tables S3-10. In general, while the consistent  
325 heterogeneity changes in development did not show any enrichment (except for miRNAs, see  
326 Supplementary Table S10), we detected a significant enrichment for the genes that become more  
327 heterogeneous during aging, with the exception that Disease Ontology terms were not significantly  
328 associated with consistent changes in either development or aging. The gene sets included specific  
329 categories such as autophagy and synaptic functions as well as broad functional categories such as  
330 regulation of transcription and translation processes, cytoskeleton or histone modifications. We also  
331 performed GSEA for each dataset separately and confirmed that these pathways show consistent  
332 patterns in aging (Supplementary Figs. S15-S19). There were 30 significantly enriched transcription  
333 factors, including *EGR* and *FOXO*, and 99 miRNAs (see Supplementary Table S8-9 for the full list). We  
334 also asked if the genes that become more heterogenous consistently across datasets are known aging-  
335 related genes, using the GenAge Human gene set<sup>36</sup>, but did not find a significant association  
336 (Supplementary Fig. S20).

337  
338 It has been reported that the total number of distinct regulators of a gene (apart from its specific  
339 regulators) is correlated with gene expression noise<sup>37</sup>. Accordingly, we asked if the total number of  
340 transcription factors (TFs) or miRNAs regulating a gene might be related to the heterogeneity change  
341 with age (Figure 6). We calculated the correlations between the total number of regulators and the  
342 heterogeneity changes and found a mostly positive (18 / 19 for miRNA and 15 / 19 for TFs), and higher  
343 correlation between change in heterogeneity and the number of regulators in aging ( $p = 0.007$  for  
344 miRNA and  $p = 0.045$  TFs). We further tested the association while controlling for the expression  
345 changes in development and aging since regulation of expression changes during development could  
346 confound a relationship. However, we found that the pattern is mainly associated with the genes that  
347 show a decrease in expression during aging, irrespective of their expression during development  
348 (Supplementary Fig. S21).

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350

351 **Figure 6.** Correlation between the change in heterogeneity and number of transcriptional regulators, i.e. miRNA  
352 and transcription factors. Each point represents a dataset, and the color shows the data source. p-values are  
353 calculated using a permutation test. The dashed line at  $y = 0$  shows zero correlation.

354

355 We further tested if genes with a consistent heterogeneity increase in aging are more central in the  
356 protein interaction network using STRING database<sup>38</sup>. Using multiple cutoffs and repeating the analysis,  
357 we observed a higher degree of interactions for the genes with increasing heterogeneity  
358 (Supplementary Fig. S22).

359

360 Johnson and Dong et al. previously compiled a list of traits that are age-related and have been  
361 sufficiently tested for genome-wide associations ( $n = 39$ )<sup>39</sup>. Using the genetic associations for GWAS  
362 Catalog traits, we tested if there are significantly enriched traits for the consistent changes in  
363 heterogeneity during aging (Supplementary Table S11). Although there was no significant enrichment,  
364 all these age-related terms had positive enrichment scores, i.e. they all tended to include genes that  
365 consistently become more heterogeneous with age during aging.

366

367 Using cell-type specific transcriptome data generated from FACS-sorted cells in mouse brain<sup>40</sup>, we also  
368 analyzed if there is an association between genes that become heterogeneous with age and cell-type  
369 specific genes, which could be expected if brain cell-type composition progressively varied among  
370 individuals with age. Although there was an overlap with oligodendrocytes and myelinated  
371 oligodendrocytes, there was no significant enrichment (which could be attributed to low power due to  
372 small overlap between aging and cell-type specific expression datasets) (Supplementary Fig. S23).

373

## 374 Discussion

375 Aging is characterized by a gradual decrease in the ability to maintain homeostatic processes, which  
376 leads to functional decline, age-related diseases, and eventually to death. This age-related  
377 deterioration, however, is thought as not a result of expression changes in a few individual genes, but  
378 rather as a consequence of an age-related alteration of the whole genome, which could be a result of  
379 an accumulation of both epigenetic and genetic errors in a stochastic manner<sup>23,41</sup>. This stochastic nature  
380 of aging impedes the identification of conserved age-related changes in gene expression from a single  
381 dataset with a limited number of samples.

382

383 In this study, we examined 19 gene expression datasets compiled from three independent studies to  
384 identify the changes in gene expression heterogeneity with age. While all datasets have samples  
385 representing the whole lifespan, we separated postnatal development (0 to 20 years of age) and aging  
386 (20 to 98 years of age) by the age of 20, as this age is considered to be a turning-point in gene  
387 expression trajectories<sup>13</sup>. We implemented a regression-based method and identified genes showing a  
388 consistent change in heterogeneity with age, during development and aging separately. At the single  
389 gene level, we did not observe significant age-related heterogeneity change in most of the datasets,  
390 possibly due to insufficient statistical power due to small sample sizes and the subtle nature of the  
391 phenomenon. We hence took advantage of a meta-analysis approach and focused on consistent  
392 signals among datasets, irrespective of their effect sizes and significance. Although this approach fails  
393 to capture patterns that are specific to individual brain regions, it identifies genes that would otherwise  
394 not pass the significance threshold due to insufficient power. Furthermore, we demonstrated that our  
395 method is robust to noise and confounding effects within individual datasets.

396

397 By analyzing age-related gene expression changes, we first observed that there are more significant  
398 and more similar changes during development than aging. Additionally, genes showing significant  
399 change during aging tended to decrease in expression (Figure 1). These results can be explained by  
400 the accumulation of stochastic detrimental effects during aging, leading to a decrease in expression  
401 levels<sup>2</sup>. Our initial analysis of gene expression changes suggested a higher heterogeneity between  
402 aging datasets.

403

404 We next focused on age-related heterogeneity change between individuals and found a significant  
405 increase in age-related heterogeneity during aging, compared to development. Notably, increased  
406 heterogeneity is not limited to individual brain regions, but a consistent pattern across different regions  
407 during aging. We found that age-related heterogeneity change is more consistent among aging  
408 datasets, which may reflect an underlying systemic mechanism. Further, a larger number of genes  
409 showed more significant heterogeneity changes during aging than in development, and the majority of  
410 these genes tended to have more heterogeneous expression.

411

412 It was previously proposed that somatic mutation accumulations<sup>2,41-43</sup> and epigenetic regulations<sup>44</sup>  
413 might be associated with transcriptome instability. While Enge et al. and Lodato et al. suggested that  
414 genome-wide substitutions in single cells are not so common as to influence genome stability and cause  
415 transcriptional heterogeneity at the cellular level<sup>23,45</sup>, epigenetic mechanisms may be relevant. Although  
416 we cannot test age-related somatic mutation accumulation and epigenetic regulation in this study, an  
417 alternative mechanism might be related to transcriptional regulation, which is considered to be  
418 inherently stochastic<sup>46</sup>. Several studies demonstrated that variation in gene expression is positively  
419 correlated with the number of TFs controlling gene's regulation<sup>37</sup>. We also found that genes with a  
420 higher number of regulators and a decrease in expression during aging become more heterogeneous.  
421 Further, significantly enriched TFs include early growth response (*EGF*), known to be regulating the  
422 expression of many genes involved in synaptic homeostasis and plasticity, and *FOXP* TFs, which  
423 regulate stress resistance, metabolism, cell cycle arrest and apoptosis. Together with these studies,  
424 our results support that transcriptional regulation may be associated with age-related heterogeneity  
425 increase during aging and may have important functional consequences in brain aging.

426

427 We next confirmed that observed increase in heterogeneity was not a result of low statistical power  
428 (Supplementary Fig. S1) or a technical artifact (Figure 3b, Supplementary Figs. S24-S25). Specifically,  
429 we tested whether increased heterogeneity during aging can be a result of the mean-variance  
430 relationship, but we found no significant effect that can confound our results. In fact, the mean-variance  
431 relationship in development and aging showed opposing profiles. We further analyzed this by grouping  
432 genes based on their expression in development and aging (Supplementary Fig. S24). The genes that  
433 decrease in expression both in development and aging showed the most opposing profiles in terms of  
434 the mean-variance relationship, which could suggest that the decrease in development are more  
435 coordinated and well-regulated whereas the decrease in aging occurs due to stochastic errors. Another  
436 potential confounder is the post-mortem interval (PMI), which is the time between death and sample  
437 collection. Since we do not have this data for all datasets we analyzed, we could not account for PMI  
438 in our model. However, using the list of genes previously suggested as associated with PMI<sup>47</sup>, we  
439 checked if the consistency among aging datasets could be driven by PMI. Only 2 PMI-associated genes  
440 were among the 147 that become consistently heterogeneous, and the distribution also suggested there  
441 is no significant relationship (Supplementary Fig. S25). We also confirmed that the increase in  
442 heterogeneity is not caused by outlier samples in datasets (Supplementary Fig. S26) or by the confound  
443 of sex with age (Supplementary Fig. S27).

444

445 One important limitation of our study is that we analyze microarray-based data. Since gene expression  
446 levels measured by microarray do not reflect an absolute abundance of mRNAs, but rather are relative  
447 expression levels, we were only able to examine relative changes in gene expression. A recent study  
448 analyzing single-cell RNA sequencing data from the aging *Drosophila* brain identified an age-related  
449 decline in total mRNA abundance<sup>48</sup>. It is also suggested that, in microarray studies, genes with lower  
450 expression levels tend to have higher variance<sup>49</sup>. In this context, whether the change in heterogeneity

451 is a result of the total mRNA decay is an important question. As an attempt to see if the age-related  
452 increase in heterogeneity is dependent on the technology used to generate data, we repeated the initial  
453 analysis using RNA sequencing data for the human brain, generated by GTEx Consortium <sup>50</sup>  
454 (Supplementary Figs. S28-30). Nine out of thirteen datasets displayed more increase than decrease in  
455 heterogeneity during aging, consistent with 18/19 microarray datasets, while the remaining four  
456 datasets showed the opposite pattern (BA24, cerebellar hemisphere, cerebellum and substantia nigra).  
457 Unlike what we observed for the microarray datasets, the change in expression levels and  
458 heterogeneity were strongly positively correlated (Supplementary Fig. S30). Unfortunately, average  
459 expression levels and variation levels in RNA sequencing is challenging to disentangle. Thus, the  
460 biological relevance of the relationship between the age-related change in expression levels and  
461 expression heterogeneity still awaits to be studied through novel experimental and computational  
462 approaches. Nevertheless, RNA sequencing analysis also suggests an overall increase in age-related  
463 heterogeneity increase.

464

465 Another limitation is related to use of bulk RNA expression datasets, where each value is an average  
466 for the tissue. While it is important to note that our results indicate increased heterogeneity between  
467 individuals rather than cells, the fact that the brain is composed of different cell types raises the question  
468 if increased heterogeneity may be a result of changes in brain cell-type proportions. To explore the  
469 association between heterogeneity and cell-type specific genes, we used FACS-sorted cell type specific  
470 transcriptome dataset from mouse brain<sup>40</sup>. We only had nine genes that have consistent heterogeneity  
471 increase and are specific to one cell-type. Eight out of nine were highly expressed in oligodendrocytes,  
472 which is consistent with the results reported in our earlier work<sup>24</sup>. However, we did not observe any  
473 significant association between cell-type specific genes and heterogeneity (Supplementary Fig. S23).  
474

475

476 Gene set enrichment analysis of the genes with increased heterogeneity with age revealed a set of  
477 significantly enriched pathways that are known to modulate aging, including longevity regulating  
478 pathway, autophagy, mTOR signaling pathway (Figure 5a). Furthermore, GO terms shared among  
479 these genes include some previously identified common pathways in aging and age-related diseases  
480 (Supplementary Figs. S16-18). We have also tested if these genes are associated with age-related  
481 diseases through GWAS, and although not significant, we found a positive association with all age-  
482 related traits defined in Johnson and Dong et al. Overall, these results indicate the effect of  
483 heterogeneity on pathways that modulate aging and may reflect the significance of increased  
484 heterogeneity in aging. Importantly, we identified genes that are enriched in terms related to neuronal  
485 and synaptic functions, such as axon guidance, neuron to neuron synapse, postsynaptic specialization,  
486 which may reflect the role of increased heterogeneity in synaptic dysfunction observed in the  
487 mammalian brain, which is considered to be a major factor in age-related cognitive decline<sup>51</sup>. We also  
488 observed genes that become more heterogeneous with age consistently across datasets are more  
489 central (i.e. have a higher number of interactions) in a protein-protein interaction network  
(Supplementary Fig. S22). Although this could mean the effect of heterogeneity could be even more

490 critical because it affects hub genes, another explanation is research bias that these genes are studied  
491 more than others.

492  
493 In summary, by performing a meta-analysis of transcriptome data from diverse brain regions we found  
494 a significant increase in gene expression heterogeneity during aging, compared to development.  
495 Increased heterogeneity was a consistent pattern among diverse brain regions in aging, while no  
496 significant consistency was observed across development datasets. Our results support the view of  
497 aging as a result of stochastic molecular alterations, whilst development has a higher degree of gene  
498 expression regulation. We also found that genes showing a consistent increase in heterogeneity during  
499 aging are involved in pathways important for aging and neuronal function. Therefore, our results  
500 demonstrate that increased heterogeneity is one of the characteristics of brain aging and is unlikely to  
501 be only driven by the passage of time starting from developmental stages.

502

## 503 **Methods**

504

### 505 **Dataset collection**

506 In this study, we performed re-analysis of publicly available transcriptome datasets to test age-related  
507 change in gene expression heterogeneity. All data collection in these previous studies were performed  
508 in accordance with relevant guidelines, regulations and approved experimental protocols, including  
509 informed consents for the use of samples for research from all donors or their next of kin.

510 *Microarray datasets*: Raw data used in this study were retrieved from the NCBI Gene Expression  
511 Omnibus (GEO) from three different sources (Supplementary Table S1). All three datasets consist of  
512 human brain gene expression data generated on microarray platforms. In total, we obtained 1017  
513 samples from 298 individuals, spanning the whole lifespan with ages ranging from 0 to 98 years  
514 (Supplementary Fig. S1).

515 *RNA sequencing dataset*: We used the transcriptome data generated by the GTEx Consortium (v6p)<sup>50</sup>.  
516 We only used the samples with a death circumstance of 1 (violent and fast deaths due to an accident)  
517 and 2 (fast death of natural causes) on the Hardy Scale excluding individuals who died of illnesses. As  
518 we focus only on the brain, we used all 13 brain tissue data in GTEx. We thus analyzed 623 samples  
519 obtained from 99 individuals.

520 *Separating datasets into development and aging datasets*: To differentiate changes in gene expression  
521 heterogeneity during aging from those during development, we used the age of 20 to separate pre-  
522 adulthood from adulthood. It was shown that the age of 20 corresponds to the first age of reproduction  
523 in human societies<sup>52</sup>. Structural changes after the age of 20 in the human brain were previously linked  
524 to age-related phenotypes, specifically neuronal shrinkage and a decline in total length of myelinated  
525 fibers<sup>3</sup>. Earlier studies examining age-related gene expression changes in different brain regions also  
526 showed a global change in gene expression patterns after the age of 20<sup>11,13,53</sup>. Thus, consistent with

527 these studies, we separated datasets using the age of 20 into development (0 to 20 years of age,  $n =$   
528 441) and aging (20 to 98 years of age,  $n = 569$ ).

529

### 530 **Preprocessing**

531 Microarray datasets: RMA correction (using the ‘oligo’ library in R)<sup>54</sup> and log2 transformation were  
532 applied to Somel2011 and Kang2011 datasets. For the Colantuoni2011 dataset, as there was no public  
533 R package to analyze the raw data, we used the preprocessed data deposited in GEO, which had been  
534 loess normalized by the authors. We quantile normalized all datasets using the ‘preprocessCore’ library  
535 in R<sup>55</sup>. Notably, our analysis focused on consistent patterns across datasets, instead of considering  
536 significant changes within individual datasets. Since we don’t expect random confounding factors to be  
537 shared among datasets, we used quantile normalization to minimize the effects of confounders, and we  
538 treated consistent results as potentially a biological signal. We also applied an additional correction  
539 procedure for Somel2011 datasets, in which there was a batch effect influencing the expression levels,  
540 as follows: for each probeset (1) calculate mean expression (M), (2) scale each batch separately (to  
541 mean = 0, standard deviation = 1), (3) add M to each value. We excluded outliers given in  
542 Supplementary Table S1, through a visual inspection of the first two principal components for the  
543 probeset expression levels (same as in Dönertaş, Fuentealba Valenzuela, Partridge, & Thornton, 2018;  
544 Dönertaş et al., 2017). We mapped probeset IDs to Ensembl gene IDs 1) using the Ensembl database,  
545 through the ‘biomaRt’ library<sup>57</sup> in R for the Somel2011 dataset, 2) using the GPL file deposited in GEO  
546 for Kang2011, as probeset IDs for this dataset were not complete in Ensembl, and 3) using the Entrez  
547 gene IDs in the GPL file deposited in GEO for the Colantuoni2011 dataset and converting them into  
548 Ensembl gene IDs using the Ensemble database, through the “biomaRt” library in R. Lastly, we scaled  
549 expression levels for genes (to mean = 0, standard deviation = 1) using the ‘scale’ function in R. Age  
550 values of individuals in each dataset were converted to the fourth root of age (in days) to have a linear  
551 relationship between age and expression both in development and aging. However, we repeated the  
552 analysis using different age scales and confirmed that the results were quantitatively similar  
553 (Supplementary Fig. S3).

554 RNA sequencing dataset: The genes with median RPKM value of 0 were excluded from the dataset.  
555 The RPKM values provided in the GTEx data were log2 transformed and quantile-normalized. Similar  
556 to the microarray data, we excluded the outliers based on the visual inspection of the first and second  
557 principal components (Supplementary Table S1). In GTEx, ages are given as 10 year intervals. We  
558 therefore used the middle point of these age intervals to represent that individual’s age.

559

560

### 561 **Age-related expression change**

562 We used linear regression to assess the relationship between age and gene expression. The model  
563 used in the analysis is:

564

$$565 (1) \quad Y_i = \beta_{i0} + \beta_{i1} * Age^{1/4} + \varepsilon_i$$

566

567 where  $Y_i$  is the scaled log2 expression level for the  $i^{\text{th}}$  gene,  $\beta_{i0}$  is the intercept,  $\beta_{i1}$  is the slope, and  $\epsilon_i$  is  
568 the residual. We performed the analysis for each dataset (development and aging datasets separately)  
569 and considered the  $\beta_1$  value as a measure of change in expression.  $p$ -values obtained from the model  
570 were corrected for multiple testing according to Benjamini and Hochberg procedure<sup>58</sup> by using 'p.adjust'  
571 function in R.

572

### 573 **Age-related heterogeneity change**

574 In order to quantify the age-related change in gene expression heterogeneity, we calculated  
575 Spearman's correlation coefficient ( $\rho$ ). The correlations were calculated between the absolute values  
576 of residuals obtained from equation (1) and the fourth root of individual age. We regarded the absolute  
577 values of residuals as a measure of heterogeneity. Therefore, high positive correlation coefficients  
578 suggest that heterogeneity increases with age, whereas strong negative correlation implies  
579 heterogeneity decreases with age.  $p$ -values were calculated from the correlation analysis and corrected  
580 for multiple testing with the Benjamini and Hochberg method using the 'p.adjust' function in R. To  
581 compare heterogeneity changes in aging and development, we employed paired Wilcoxon test  
582 ('wilcox.test' in the R 'stats' package) in which we compared median heterogeneity changes in aging  
583 and development dataset pairs.

584

### 585 **Principal Component Analysis**

586 We conducted principal component analysis on both age-related changes in expression ( $\beta$ ) and  
587 heterogeneity ( $\rho$ ). We followed a similar procedure for both analyses, in which we used the 'prcomp'  
588 function in R. The analysis was performed on a matrix containing  $\beta$  values (for the change in expression  
589 level) and  $\rho$  values (for the change in heterogeneity), for 11,137 commonly expressed genes for all 38  
590 development and aging datasets. In each dataset, the estimates of expression change ( $\beta$ ) or  
591 heterogeneity change ( $\rho$ ) values were scaled for each dataset before calculating principal components.

592

### 593 **Permutation test**

594 We performed a permutation test, taking into account the non-independence of samples across the  
595 Somel2011 and Kang2011 datasets, due to the fact that these datasets include multiple samples from  
596 the same individuals for different brain regions. We first randomly permuted ages among individuals,  
597 not samples, for 1,000 times in each data source, using the 'sample' function in R. Next, we assigned  
598 ages of individuals to corresponding samples and calculated age-related expression and heterogeneity  
599 change for each dataset, corresponding to different brain regions. For the tests related to the changes  
600 in gene expression with age, we used a linear model between gene expression levels and the  
601 randomized ages. In contrast, for the tests related to the changes in heterogeneity with age, we  
602 measured the correlation between the randomized ages and the absolute value of residuals from the  
603 linear model that is between expression levels and non-randomized ages for each gene. In this way,

604 we preserved the relationship between age and expression, and we were able to ensure that our  
605 regression model was viable for calculating age-related heterogeneity change. Using expression and  
606 heterogeneity change estimates calculated using permuted ages, we tested (a) if the correlation of  
607 expression (and heterogeneity) change in aging is higher than in development datasets; (b) if the  
608 correlations of expression (and heterogeneity) change in development and in aging datasets are  
609 significantly higher than null expectation; (c) if the number of genes showing significant change in  
610 expression (and heterogeneity) is significantly higher in aging than in development datasets; (d) if the  
611 overall increase in age-related heterogeneity during aging is significantly higher than development; (e)  
612 if the observed consistency in heterogeneity increase is significantly different from expected. All tests  
613 using permuted ages were performed one-tailed. We also demonstrate that our permutation strategy is  
614 more stringent than random permutations in Supplementary Figure S10, giving the distributions  
615 calculated using both dependent permutations and random permutations.

616

617 To test the overall correlation within development or aging datasets for the changes in expression ( $\beta$ )  
618 and heterogeneity ( $\rho$ ), we calculated median correlations among independent three subsets of datasets  
619 (one Kang2011, one Somel2011 and the Colantuoni2011 dataset), taking the median value calculated  
620 for each possible combination of independent subsets ( $16 \times 2 \times 1 = 32$  combinations). Using 1,000  
621 permutations of individuals' ages, we generated an expected distribution for the median correlation  
622 coefficient for triples and compared these with the observed values, asking how many times we observe  
623 a higher value. We used this approach to calculate expected median correlation among development  
624 (and aging) datasets, because the number of independent pairwise comparisons are outnumbered by  
625 the number of dependent pairwise comparisons, causing low statistical power.

626

627 To further test the significance of the difference between correlations among development and aging  
628 datasets, we calculated the median difference in correlations between aging and development datasets  
629 for each permutation. We next constructed the null distribution of 1,000 median differences and  
630 calculated empirical  $p$ -values comparing the observed differences with these null distributions. Next, to  
631 test the significance of the difference in the number of significantly changing genes between  
632 development and aging, we calculated the difference in the number of genes showing significant  
633 change between development and aging datasets for each permutation. Empirical  $p$ -values were  
634 computed according to observed differences. Likewise, to test if the overall increase in age-related  
635 heterogeneity during aging is significant compared to development, we computed median differences  
636 between median heterogeneity change values of each aging and development dataset, for each  
637 permutation, followed by an empirical  $p$ -value calculation to answer if the aging datasets have a higher  
638 increase in age-related heterogeneity.

639

640 **Expected heterogeneity consistency**

641 Expected consistency in heterogeneity change was calculated from heterogeneity change values  
642 ( $\rho$ ) measured using permuted ages. For each permutation, we first calculated the total number of genes  
643 showing consistent heterogeneity increase for N number of datasets (N = 0, ..., 19). To test if observed  
644 consistency significantly differed from the expected, we compared observed consistency values to the  
645 distribution of expected numbers, by performing a one-sided test for the consistency in N number of  
646 datasets, N = 1, ..., 19.

647

#### 648 **Clustering**

649 We used the k-means algorithm ('kmeans' function in R) to cluster genes showing consistent  
650 heterogeneity change (n=147) according to their heterogeneity profiles. We first took the subset of the  
651 heterogeneity levels (absolute value of the residuals from equation (1)) to include only the genes that  
652 show a consistent increase with age and then scaled the heterogeneity levels to the same mean and  
653 standard deviation. Since the number of samples in each dataset is different, just running k-means on  
654 the combined dataset would not equally represent all datasets. Thus, we first calculated the spline  
655 curves for scaled heterogeneity levels for each gene in each dataset (using the 'smooth.spline' function  
656 in R, with three degrees of freedom). We interpolated at 11 (the smallest sample size) equally distant  
657 age points within each dataset. Then we used the combined interpolated values to run the k-means  
658 algorithm with k = 8, a liberal choice, given the total number of genes being 147.

659

660 To test association of the clusters with Alzheimer's Disease, we retrieved overall AD association scores  
661 of the 147 consistent genes (n = 40) from the Open Targets Platform<sup>59</sup>.

662

#### 663 **Functional Analysis**

664 We used the "clusterProfiler" package in R to run Gene Set Enrichment Analysis, using Gene Ontology  
665 (GO) Biological Process (BP), GO Molecular Function (MF), GO Cellular Compartment (CC),  
666 Reactome, Disease Ontology (DO), and KEGG Pathways. We performed GSEA on all gene sets with  
667 a size between 5 and 500, and we corrected the resulting *p*-values with the Benjamini and Hochberg  
668 correction method. To test if the genes with a consistent increase or decrease in their expression are  
669 associated with specific functions, we used the number of datasets with a consistent increase to run  
670 GSEA. Since we are running GSEA using number of datasets showing consistency, our data includes  
671 many ties, potentially making the ranking ambiguous and non-robust. In order to assess how robust our  
672 results are, we ran GSEA 1,000 times on the same data and counted how many times we observed the  
673 same set of KEGG pathways as significant (Supplementary Table S3). The lowest number among the  
674 pathways with a significant positive enrichment score was 962 out of 1,000 (Phospholipase D signaling  
675 pathway). Moreover, we repeated the same analysis using the heterogeneity change levels ( $\rho$ ), instead  
676 of using the number of datasets with a consistent change, for each dataset to confirm the gene sets are  
677 indeed associated with the increase or decrease in heterogeneity (Supplementary Figs. S15-S19). We  
678 visualized the KEGG pathways using 'KEGGgraph' library in R and colored the genes by the number  
679 of datasets that show an increase.

680

681 We also performed an enrichment analysis of the transcription factors and miRNA to test if specific TFs  
682 or miRNAs regulate the genes that become more heterogeneous consistently. We collected gene-  
683 regulator association information using the Harmonizome database<sup>60</sup>, “MiRTarBase microRNA Targets”  
684 (12086 genes, 596 miRNAs) and “TRANSFAC Curated Transcription Factor Targets” (13216 genes,  
685 201 TFs) sets. We used the ‘fgsea’ package in R, which allows GSEA on a custom gene set. We tested  
686 the association for each regulator with at least 10 and at most 500 targets. Moreover, we tested if the  
687 number of regulators is associated with the change in heterogeneity. We first calculated the correlation  
688 between heterogeneity change with age (or the number of datasets with an increase in expression  
689 heterogeneity) and the number of TFs or miRNAs regulating that gene, for aging and development  
690 separately. We repeated the analysis while accounting for the direction of expression changes in these  
691 periods (*i.e.* separating genes into down-down, down-up, up-down, and up-up categories based on their  
692 expression in development and aging, Supplementary Fig. S21). To test the difference in the  
693 correlations between aging and development, we used 1,000 random permutations of the number of  
694 TFs. For each permutation, we randomized the number of TFs and calculated the correlation between  
695 heterogeneity change (or the number of datasets with an increase in heterogeneity) and the randomized  
696 numbers. We then calculated the percentage of datasets where aging has a higher correlation than  
697 development. Using the distribution of percentages, we tested if the observed value is expected by  
698 chance.

699

#### 700 **Protein-protein interaction network analysis**

701 We downloaded all human protein interaction data from the STRING database (v11)<sup>38</sup>. Ensembl  
702 Peptide IDs are mapped to Ensembl Gene IDs using the “biomaRt” package in R. Here we aimed to  
703 test whether genes showing consistent increase in heterogeneity have a different number of interactors  
704 than other genes. For this we calculated the degree distributions for the genes that become consistently  
705 more heterogeneous with age and all remaining genes using different cutoffs for interaction confidence  
706 scores. In order to calculate the significance of the difference, we i) calculated the number of interactors  
707 (degree) for each gene, ii) for 10,000 times, randomly sampled k genes from all interactome data (k =  
708 number of genes that become heterogeneous with age across all datasets and have interaction  
709 information in STRING database, after filtering for a cutoff), iii) calculated the median of degree for each  
710 sample. We then calculated an empirical *p*-value by asking how many of these 10,000 samples we see  
711 a median degree that is equivalent to or higher than our original value. The number of genes and  
712 interactions after each cutoff are given in Supplementary Figure S22.

713

#### 714 **Cell-type specificity analysis**

715 Using FACS-sorted cell-type specific transcriptome data from the mouse brain<sup>40</sup>, we checked if there is  
716 any overlap between genes that become heterogeneous with age and cell-type specific genes. We  
717 downloaded the dataset from the GEO database (GSE9566) and preprocessed it by performing: i) RMA  
718 correction using the ‘affy’ package in R<sup>61</sup>, ii) log2 transformation, iii) quantile normalization using the

719 'preprocessCore' package in R<sup>55</sup>, iv) mapping probeset IDs to first mouse genes, and then human  
720 genes. We only included genes that have one to one orthologs in humans, after filtering out probesets  
721 that map to multiple genes. We defined cell-type specific genes by calculating the effect size (Cohen's  
722 D) for each gene and cell type and identifying genes that have an effect size higher than or equal to 2  
723 as specific to that cell type. At this cutoff, there was no overlap between cell type specific gene lists. To  
724 test for association between heterogeneity and cell type specificity, we used the Fisher's exact test  
725 using the R 'fisher.test' function.

726

## 727 **Code Availability**

728 All analysis was performed using R and the code to calculate heterogeneity changes with age is  
729 available as an R package 'hetAge', documented at <https://mdonertas.github.io/hetAge/>. "ggplot2"<sup>62</sup> and  
730 "ggpubr"<sup>63</sup> R libraries were used for the visualization.

731

## 732 **Data availability**

733 We performed re-analysis of the raw data that we downloaded from the GEO database (GSE30272,  
734 GSE25219, GSE22569, GSE18069) and GTEx data portal. All results generated in this study are  
735 available as Supplementary Tables and all summary statistics are available in the BioStudies database  
736 (<http://www.ebi.ac.uk/biostudies>) under accession number S-BSST273.

737

## 738 **Author Contributions**

739 H.M.D. conceived and designed the study with the contributions from M.S., and J.M.T.. U.I. and H.M.D.  
740 analyzed the data. U.I. and H.M.D. interpreted the results and wrote the manuscript with the  
741 contributions from M.S. and J.M.T. All authors read, revised and approved the final version of this  
742 manuscript.

743

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747

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750

## 751 **Competing Interests**

752 The authors declare no competing interests.

753

754

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